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The Effect of Nerve Growth Factor (NGF) Incorporation into Swine Intestinal Submucosa (SIS) Suture Material on the Healing Process in Gastrocnemius Muscle

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PHILADELPHIA COLLEGE OF OSTEOPATHIC MEDICINE

THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

THE EFFECT OF NERVE GROWTH FACTOR (NGF) INCORPORATION INTO SWINE INTESTINAL SUBMUCOSA (SIS) SUTURE MATERIAL ON THE HEALING PROCESS IS GASTROCNEMIUS MUSCLE

A Thesis in Biomedical Sciences by Nicole K. Alexander, D.O.

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Submitted in Partial Fulfillment of the Requirements for the

Degree of Masters of Biomedical Sciences

May 2013

We approve the thesis of Nicole K Alexander, D.O.

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Professor of Histology and Anatomy

Abstract

The repair of gastrocnemius muscle incisions were evaluated histologically in a rat model. Comparisons were made between carbon dioxide laser and scalpel incisions closed with sutures prepared from Swine Intestinal Submucosa (SIS) with and without added Nerve Growth Factor. This study was conducted in conjunction with two other studies, one comparing epidermal repair and the other comparing kinesthetic changes post-operatively in the same animal model. Thirty-five days post-surgery the animals were euthanized and an area of muscular tissue encompassing the operative site was excised and evaluated microscopically for the following: presence of macrophages, integrity of the muscle, leukocytes present within the muscle, presence of vasculature within the muscle, and granulation tissue width. The evaluation of healing as reflected by degree of muscle integrity demonstrated that added growth factor improved healing regardless of modality while the laser plus growth factor demonstrated the highest degree of muscle integrity. While the increase in vasculature was greatest for the healed scalpel incisions, the laser groups in general showed greater increases in the width of the muscle, number of macrophages. All four groups had an increased number of leukocytes. This study and previous studies have shown that muscle regeneration is significantly increased with the addition of GF. The differences observed between these two surgical techniques suggest that one or the other can be appropriately selected depending upon the characteristics of the repair goals that are being sought. The full potential for clinical application of these repair modalities will only be realized with additional studies, which explore the healing mechanism to a greater depth and for many different types of tissues.

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I would like to thank my fabulous thesis advisor, Dr. Charlotte Greene for her encouragement, patience, and countless hours of work with me. I would also like to thank Dr. Marcus Bell for his help with analyzing, documenting, and editing the results. Finally, I would like to thank Mr. David Cavanagh for his help interpreting the data and the results. Thank you also to Dr. Bruce Stouch and Dr. Gwendolyn Harley. I am forever indebted to my parents Mr. and Dr. Robert Alexander, who have been supportive of my education since the very beginning. Lastly, I would like to thank my wonderful husband Mr. Chidozie Anyaogu for suffering quietly while I complained and for being my practice audience for hours on end.

Chapter 1. INTRODUCTION

Background

This study was part of a series of studies designed to explore various techniques to improve skeletal muscle wound healing. This study compared and contrasted how CO_2 laser or scalpel incisions affected gastrocnemius muscle healing in a rat model, as well as the effect of different types of suture material, and most importantly how the effect of added exogenous nerve growth factor (NGF) affected healing in this tissue. Sutures prepared from swine intestinal submucosa (SIS) or commercial 3-0 vicryl (Ethicon; Cincinnati, Ohio) sutures were utilized to repair the incisions and exogenous nerve growth factor (NGF) was added to the incision site to supplement the endogenous growth factors found in native SIS.

Current Research

There are a number of different focuses in studies reported in the literature regarding muscle healing over the past decade. Cyclooxygenase (COX) Inhibitors have been studied and have been proven to decrease muscle regeneration by promoting fibrosis (1).

Myostatin, also known as Growth differentiation factor 8, has been studied in mysostatin null mice and has been proven to be a negative regulator of muscle regeneration and also prevents migration of myoblasts and macrophages to the site of injury (2).

Matrix Metalloproteinases (MMP) are proteins that are part of the exogenous matrix. Their function is to aid in digesting fibrous tissue and releasing local growth

factors. Addition of MMP's has shown an increase in muscle regeneration, however it has not shown restoration of function to the injured tissue (3).

Muscle-derived stem cells are currently being researched to aid in muscle healing and regeneration. One trial has resulted in successful cases over a one-year period following the implantation of these cells to restore detrusor muscle function, with 5 out of 8 females reporting improvements, one achieving complete continence, and none sustaining any adverse outcomes (4).

Studies are also being done regarding muscular dystrophy and preventing muscle wasting. This year a study was done targeting Protein Kinase C (PKC) and whether or not it could prevent the inflammatory response and disease progression in a muscular dystrophy. They found that muscle wasting was greatly prevented in those mice that had their PKC targeted, while muscle regeneration, maintenance and performance was significantly improved in those same mice (5).

The effects of testosterone on the skeletal muscle regeneration of young (2-month-old) and aged (24-month-old) mice were also tested. The results showed that testosterone increases the number of proliferating satellite cells in both young and aged castrated animals. Testosterone supplementation increases the number and the cross-sectional area of regenerating fibers. Testosterone increases satellite cell activation, proliferation and the regeneration of both young and aged mouse muscle (6).

Muscle Healing

There are three phases involved in the healing of injured skeletal muscle. The first phase is the destruction phase, it is characterized by hematoma formation,

followed by muscle necrosis, degeneration, and an inflammatory cell response. The repair phase includes phagocytosis of the damaged tissue, regeneration of the striated muscle, production of a connective-tissue scar and capillary ingrowth. In the final remodeling phase, the regenerated muscle matures and contracts with reorganization of the scar tissue. There is often incomplete restoration of the functional capacity of the injured muscle (7).

The rat gastrocnemius is a powerful skeletal muscle with fascicles that attach obliquely to the dorsal aspect of the lower hind limb. Along with the soleus muscle it forms the calf muscle. It participates in standing, jumping, running, and walking, functioning to plantar flex the foot at the ankle and flex the leg at the knee joint. A severe muscle flexion can result in injury that leads to a muscle tear or strain that can be acutely painful and disabling. The rat gastrocnemius is also one of the most common muscles to experience muscle spasms, which are painful, involuntary contractions of the muscle that may last several minutes and are very similar to those experienced in the human gastrocnemius; this is why it was used as an experimental model in this study (8).

Carbon Dioxide Laser

The CO_2 laser is a surgical instrument that can be applied precisely and accurately and has a high degree of absorption in soft tissue with limited damage to the surrounding areas, which enables it to be utilized as a beneficial alternative to the traditional scalpel (9).

The CO_2 laser has many advantages over the scalpel including the following: less bleeding - as it cuts the laser seals small blood vessels; less pain to the patients - the laser beam seals nerve endings and lymphatics, resulting in less edema and pain; reduced risk of infection - kills bacteria that is in its path therefore producing a sterile field; quicker recovery time - the advantages previously listed aid in this (reduced risk of infection, less bleeding, and less pain) in addition to less swelling.

Growth Factors (GFs)

Growth factors are soluble proteins that regulate mitosis, cell differentiation, cell proliferation, and programmed cell death (7,10,11,12). A large number of growth factors are specific to the wound healing process, but only a few that have been identified thus far as being involved in muscle healing. The growth factors that have been demonstrated to be involved in the healing process of skeletal muscle are basic fibroblast growth factor (b-FGF), insulin growth factor type 1 (IGF-1) and nerve growth factor (NGF). In the aforementioned study b-FGF, IGF-1, and NGF were proven to be potent stimulators of proliferation and fusion of myoblasts in vitro (13,14).

b-FGF is found in the basement membranes and in the subendothelial extracellular matrix of blood vessels. TGFb is a growth factor that can regulate angiogenesis and promote regenerative matrix formation in healing wounds (15, 16,17). FGF2 stimulates proliferation of endothelial cells and angiogenesis (12,15,20,21). VEGF has a major role in creating new blood vessels, during the development of the embryo, new blood vessels after injury, muscle following exercise, and collateral circulation to bypass blocked vessels (15, 22,23).

It has been postulated that growth factors aid in healing muscle in vivo (7,15). Specifically research has shown that Insulin Growth Factor 1 (IGF-1) and b-FGF have the ability to accelerate healing in muscle, however the results regarding Nerve Growth Factor (NGF) were inconclusive (7). This is precisely why NGF was chose for this study. NGF is shown to be a potent stimulator of myoblast differentiation, growth, and maturation in vitro (7,15).

Small Intestine Submucosa (SIS)

SIS is a naturally derived biomaterial from the small intestine of pigs. To prepare the submucosa, the mucosal, serosal, and muscular layers of the intestine must be removed, leaving a very durable collagenous layer; the submucosa. The SIS is composed of a strong extracellular matrix of proteoglycans and glycoproteins (18,19). SIS is currently being studied in wound repair and healing in hernia repair, dermal wound healing, and vascular grafts just to name a few applications (12,13,19,24,25). The main benefit of using SIS is that it is acellular and does not provoke the intense immune response seen with the use of many foreign materials and tends to work with the intrinsic body processes that stimulate the surrounding tissue to grow (11,12,19,24,25). SIS has a significant growth factor content (19,21,22), which stimulates cell division, migration and differentiation. The growth factors found in the SIS membrane are basic fibroblast growth factor (b-FGF), transforming growth factor β (TGFb) and vascular endothelial growth factor (VEGF) (15, 22, 26, 27).

Rationale for the Present Study

We focused on the histological effects that NGF has on myoblast differentiation and proliferation, the number of myofibers, and the number the fibroblasts that were present. We compared on the basis of whether or not NGF was added. Previous studies have noted that NGF may have some role in muscle regeneration and this study was designed to support or refute this claim.

Chapter 2. MATERIALS AND METHODS

Animal Model

The experimental protocol described in this study was approved by the PCOM institutional animal care and utilization committee in accordance with National Institutes of Health and the United States Department of Agriculture guidelines for the use and care of laboratory animals. Animals were housed in the animal resources facility in a room with controlled temperature (20 –22°C) on a 12-hr light/dark cycle and were provided rat chow and water ad libitum. At the end of the experimental protocol, each rat was euthanized by CO2 inhalation.

The Sprague Dawley rat is an outbred multipurpose breed of albino rat used extensively in medical research. Its main advantage is its calmness and ease of handling. This breed of rat was first produced by the Sprague Dawley farms (later to become the Sprague Dawley Animal Company, Madison, WI). These rats were first bred in 1925. The average litter size of the Sprague Dawley rat is 10.5. In this rat model, 1-mo old rats are considered juvenile animals (34). The adult body weight is 250–300 g for females, and 450–520 g for males. The typical life span is 2.5–3.5 years. The rats used in this study were approximately 1 month old and therefore their body weight was a little smaller ranging from 310-365 g.

Preparation of SIS sutures

Porcine small intestine was obtained from a USDA-approved vendor in the fresh state. The jejunum was identified and immediately separated from the rest of the small intestine, keeping a remnant of the mesentery intact to distinguish orientation. A

segment of jejunum was transected and cut longitudinally along the remnant of the mesentery, forming a sheet. The sheet was placed serosal side up. Starting at the edge of the sheet, the serosa and muscularis were peeled away from the submucosa using forceps and discarded. The mucosal surface was exposed and denuded. The remaining SIS sheet was further subdivided into thin strips to be used as suture material. The final dimension of the suture material was comparable to a 7-0 commercial suture material. Sutures were prepared the day before surgery (and sterilized in a 10% gentamicin/physiological saline solution). Sutures were threaded to a C-3 reverse cutting needle the day before surgery and returned to the sterilizing solution.

Preparation of NGF

NGF beta Mouse was used in this study (Prospec Protein Specialists, Rehovot, Israel). It is produced in the submaxillary gland of the adult mouse. It is a homodimer, non-glycosylated, polypeptide chain containing 2 identical 120 amino acids and having a molecular mass of 13,471 Dalton each. The NGF beta Mouse was purified by advanced biology purification technology.

The lyophilized Mouse NGF beta was reconstituted in sterile 19 MΩ-cm H_2O that was further diluted to 0.1 ml of a solution containing 1000 ng of NGF per 1 ml of saline so it could then be subsequently added to other aqueous solutions.

Operative Technique

1. 32 male Sprague-Dawley rats, weighing between 310 and 365 grams were randomly assigned to one of four groups.

2. All animals were induced to a surgical plane of anesthesia with an intramuscular (IM) injection of Ketamine 40mg/kg and Xylazine 5mg/kg.
3. Mezlocillin sodium 75mg/kg IM was administered both pre & post-operatively for infection control.
4. A posterior longitudinal skin incision was made along the left calf to expose the surgical field using either CO_2 Laser (Groups A and B) or Scalpel (Groups C and D), and in the groups that used that laser 5 watts was used in continuous mode for 1 second x 4 passes.
5. The gastrocnemius muscle was dissected and lacerated by cutting 60% of the length from the distal insertion, 75% of the width, and 50% of the thickness using either CO_2 Laser (Groups A and B) or Scalpel (Groups C and D).
6. In the muscle SIS was used to close the incision using the modified kessler technique. In the skin SIS and 3-0 vicryl was used to close the incisions.

Post-operative Protocol

1. An initial dose of Butorphanol (2.0 mg/kg, SQ) was administered 30 minutes prior to emergence from anesthesia for post operative pain and was followed up with another 4 hours later.
2. Each incision was washed and dried aseptically, and Neosporin® ointment applied.
3. The rats were returned to the animal colony after recovery from anesthesia and stabilization of vital signs.
4. They were maintained on normal food and watering schedules, and were permitted unrestricted cage activity.
5. Each rat was observed once daily for the first 10 days after the surgery, and neosporin ointment applied to the incision line as needed, the incision was inspected for cleanliness, dryness and closure, and their feeding habits and activity were noted.
6. Supplimental injections of growth factor were injected into the incision line on days 1, 3, 5, and 10 post surgery (Groups B and D).
7. Each animal was euthanized 35 days post-operatively by CO2 inhalation. Death was confirmed by observation of the heart in stasis.
8. An area of cutaneous and muscular tissue sample encompassing the operative site was removed and placed in formalin, paraffin embedded, and sectioned according to standard histological techniques.
9. The remains were disposed of by a commercial vendor according to institutional policy.

Tissue Preparation

An area of cutaneous tissue encompassing the operative site was excised, formalin fixed, paraffin embedded, sectioned, and stained by Toren's Method for mast cell granules, collagen and cartilage, muscle and elastic fibers, fibrin, bone, colloid, keratin and erythrocytes. Imaging was visualized and photographed using a Nikon Eclipse 50i Microscope. The sections were analyzed using NIS-elements AR 3.0 to quantitate the parameters of interest.

Tissue Analysis

1. Macrophages near the SIS suture: were analyzed by counting the cell number in a 40x objective using a scale of 0 to 3 in which 1 was equal to a count of 1 to 4, 2 of 5 to 10, 3, a count of more than 10 cells.
2. Macrophages near the scalpel incision: were analyzed by counting the cell number in a 40x objective using a scale of 0 to 3 in which 1 was equal to a count of 1 to 4, 2 of 5 to 10, 3, a count of more than 10 cells.
3. Macrophages near the laser incision: were analyzed by counting the cell number in a 40x objective using a scale of 0 to 3 in which 1 was equal to a count of 1 to 4, 2 of 5 to 10, 3, a count of more than 10 cells.
4. Fibroblasts: were analyzed by counting the cell number in a 40x objective using a scale of 0 to 3 in which 1 was equal to a count of 1 to 4, 2 of 5 to 10, 3, a count of more than 10 cells.
5. Neutrophils: were analyzed by counting the cell number in a 40x objective using a scale of 0 to 3 in which 1 was equal to a count of 1 to 4, 2 of 5 to 10, 3, a count of more than 10 cells.
6. Leukocytes: were analyzed by counting the cell number in a 40x objective using a scale of 0 to 3 in which 1 was equal to a count of 1 to 4, 2 of 5 to 10, 3, a count of more than 10 cells.
7. Integrity of the Muscle: the organized/disorganized appearance was visualized at 20X and was rated on a scale of 0-3, 0 for "disorganized," through 3 for "highly organized." Muscle Integrity was measured by looking at several parameters; the borders: whether they were well-defined or have ragged edges; texture: was it

consistent or inconsistent; uniformity throughout the myocytes without holes or breaks or different colors throughout with holes and/or detached nuclei.

8. Granulation Tissue: the width was measured in pixels by drawing a line that extended from point A to point B at 40X magnification and then converted and reported in microns.
9. Vasculature: the number in each 20X field was counted and recorded.

Statistical Methods

A maximum number of animals per group of 8 was specified for this study (N=32). This study is powered to detect a relatively large effect among the 4 groups. A one-way design with 4 groups has sample sizes of 2, 2, 2, and 2. The null hypothesis is that the standard deviation of the group means is 0.0 and the alternative standard deviation of the group means is 8.7. The total sample of 8 subjects achieves a power of 0.244 using the F-Test with a target significance level of 0.050 and an actual significance level of 0.048. The average within group standard deviation assuming the alternative distribution is 10.0. These results are based on 2000 Monte Carlo samples from the null distributions: Normal(M0S); Normal(M0 S); Normal(M0 S); and Normal(M0 S) and the alternative distributions: Normal(M1S); Normal(M0 S); Normal(M0 S); and Normal(M0 S). Other parameters used in the simulation were: M0 = 10.0, M1 = 30.0 and S = 10.0 (30,31, 32).

The overall analysis that mirrors the design of the study was a one-factor (randomized treatment assignment) random-effects generalized linear model. For example, qualitative measures scored on a 0-4 scale from the histopathology were compared between groups specifying a multinomial distribution. The distribution for variables such as granulation tissue were compared between groups specifying a continuous distribution. When the results from the overall model for a parameter were

significant ($p<0.05$), then a post hoc test (eg. SNK) was used to determine the ranking of the 4 groups to understand which groups differ from one another.

Chapter 3. RESULTS

Muscle Integrity

Muscle Integrity was measured by looking at several parameters; the borders: whether they were well-defined or have ragged edges; texture: was it consistent or inconsistent; uniformity throughout the myocytes without holes or breaks or different colors throughout with holes and/or detached nuclei.

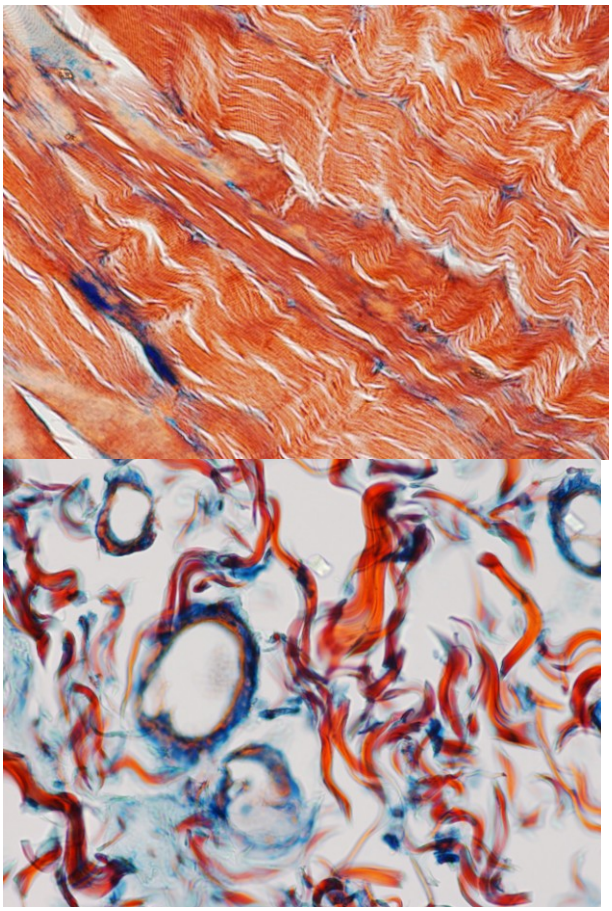

Figure 1. A. Light micrograph of Organized Muscle (40x magnification; laser plus GF)
Figure 1. B. Light micrograph of Disorganized muscle (40x magnification; laser no GF)

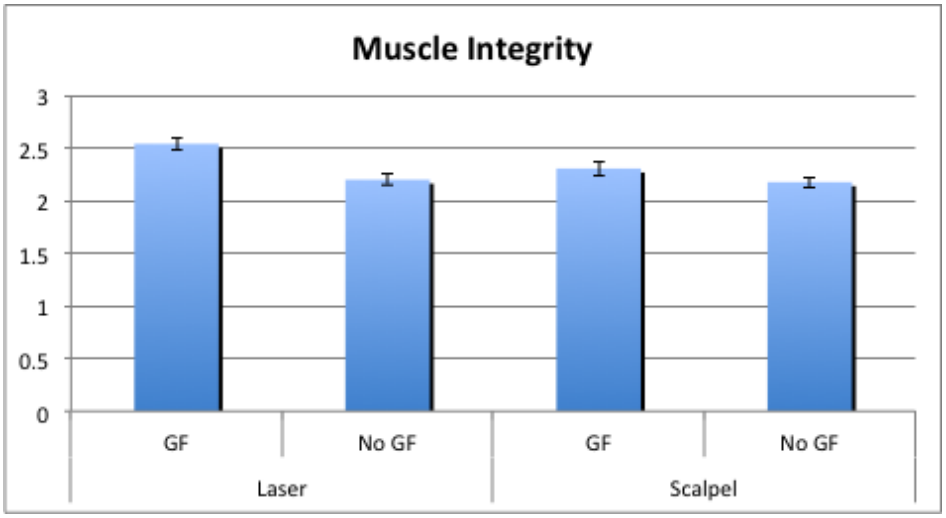

Figure 2. Muscle integrity in all groups are compared. Laser with and without growth factor (n=85; n=85), scalpel with and without growth factor (n=85; n= 85). The Laser with growth factor group had the highest degree of muscle integrity followed by the scalpel with growth factor group.

When the laser and scalpel groups with added growth factor and laser group without added growth factor (n=8; n=8; n=7, respectively) were compared, the muscle integrity of the laser group with added growth factor had a higher degree of muscle integrity than the scalpel group with added growth factor and the laser group without added growth factor ($p<0.082$). The scalpel group with added growth factor had a higher degree of muscle integrity than the laser group without added growth factor ($p<0.07$). The laser group without added growth factor

The results showed that the laser plus growth factor group had the highest degree of muscle integrity followed by the scalpel plus growth factor group, then the laser without growth factor group, and lastly the scalpel without growth factor.

Vasculature

Vasculature was analyzed by counting the number of blood vessels in a 20x objective using a scale of 0 to 2 in which 0 was no blood vessels, 1 was equal to 1 to 4 blood vessels, and 2 was equal to 5 or more blood vessels per slide.

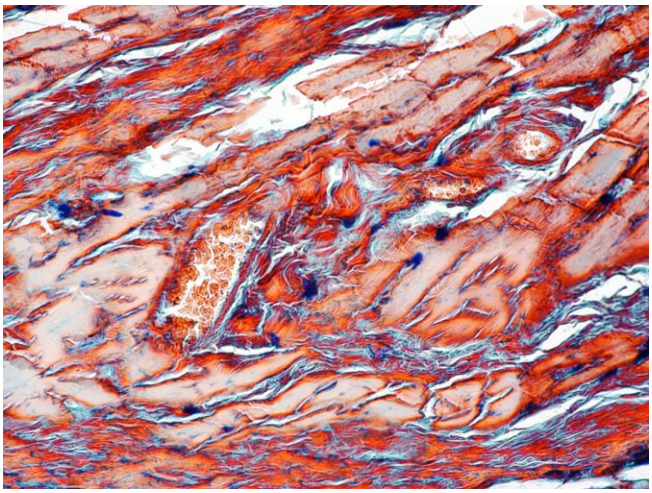

Figure 3. Light Micrograph of Muscle with blood vessels (20x magnification; scalpel plus GF)

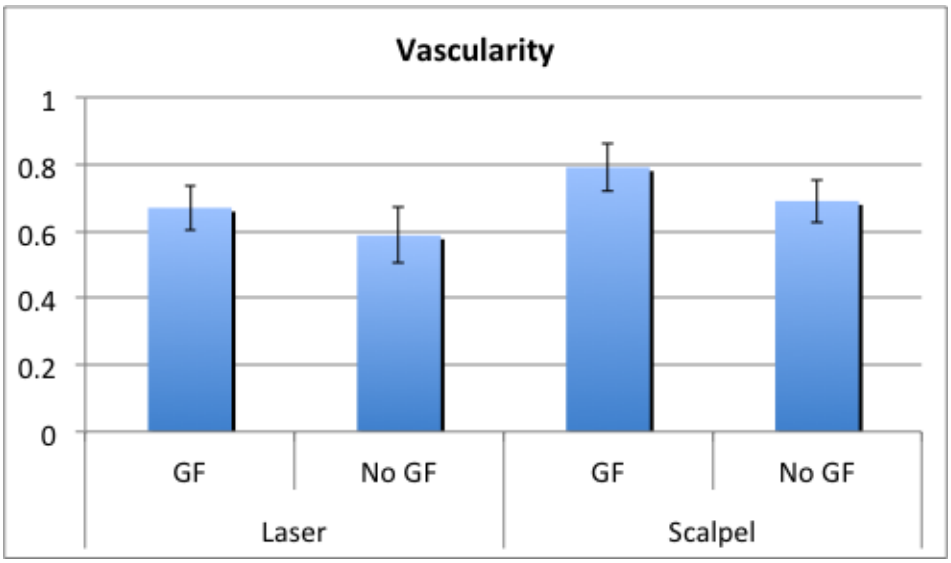

Figure 4. Vascularity in all groups are compared. Laser with and without growth factor (n=91, n=97), scalpel with and without growth factor (n=96, n= 97) are compared. The scalpel with growth factor group had the highest degree of muscle integrity followed by the scalpel without growth factor group, then the laser with growth factor, and lastly the laser without growth factor group.

The results for vascularity showed that when comparing all four experimental groups the scalpel plus growth factor group had the highest degree of vascularity. The scalpel without growth factor group followed, then the laser with growth factor group, and lastly the laser without growth factor group.

Granulation Tissue

Sutures are seen as foreign bodies by all types of tissue, including the muscle and are thought to cause a local and possibly distant, immunologic reaction reflected by the development of granulation tissue, which is part of the normal wound healing process. The width of the granulation layer was measured in this analysis in order to show the extent of injury and how it varied between the groups using additional growth factor and between the surgical methods.

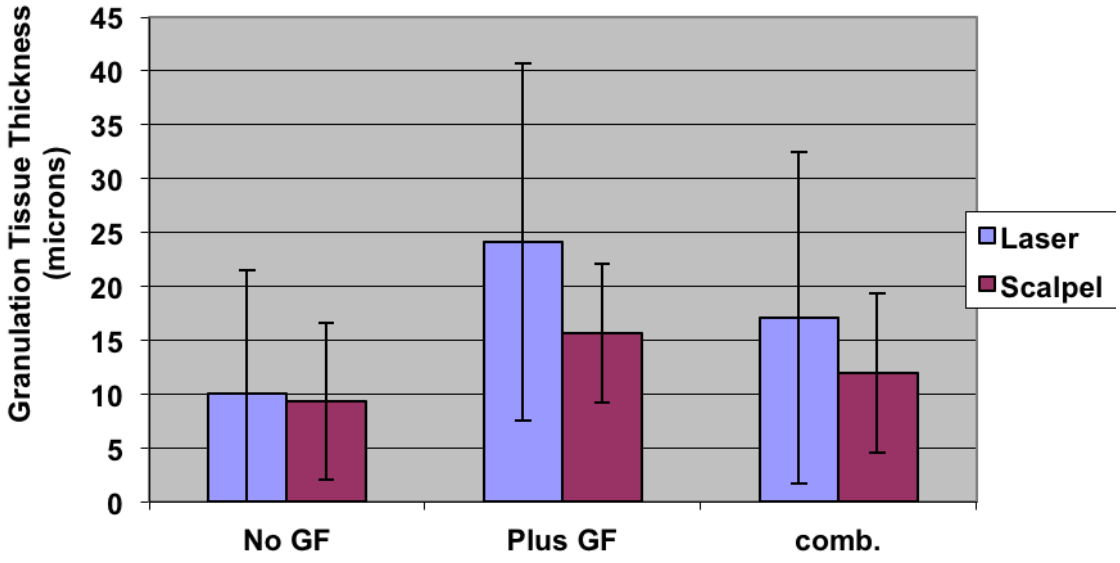

Figure 5. The laser incision repair resulted in a significantly greater thickness of the granulation tissue layer surrounding the suture holes in the muscle (n=12) than the scalpel (n=12) incision ($p<0.002$).).

All Results

PARAMETER NUMBER	PARAMETER DESCRIPTION	MEDIAN INTRA-ANIMAL SCORE	METHOD: L = LASER, S = SCALPEL	COUNT	PERCENT	P-VALUE
1	Macrophages near SIS suture 0 - 3	0	L	12	100.00	
1	Macrophages near SIS suture 0 - 3	0	S	12	100.00	
2	Macrophages near commercial suture 0 - 3	0	L	12	100.00	
2	Macrophages near commercial suture 0 - 3	0	S	12	100.00	
3	Macrophages near scalpel incision 0 - 4	0	L	12	100.00	<0.001
3	Macrophages near scalpel incision 0 - 4	0	S	0	0.00	
3	Macrophages near scalpel incision 0 - 4	1	L	0	0.00	<0.001
3	Macrophages near scalpel incision 0 - 4	1	S	8	66.67	
3	Macrophages near scalpel incision 0 - 4	2	L	0	0.00	<0.001
3	Macrophages near scalpel incision 0 - 4	2	S	4	33.33	
4	Macrophages near laser incision 0 - 4	0	L	0	0.00	<0.001
4	Macrophages near laser incision 0 - 4	0	S	12	100.00	
4	Macrophages near laser incision 0 - 4	1	L	6	50.00	<0.001
4	Macrophages near laser incision 0 - 4	1	S	0	0.00	
4	Macrophages near laser incision 0 - 4	2	L	6	50.00	<0.001
4	Macrophages near laser incision 0 - 4	2	S	0	0.00	
5	Muscle integrity 0 - 3	1	L	0	0.00	0.043
5	Muscle integrity 0 - 3	1	S	1	8.33	
5	Muscle integrity 0 - 3	2	L	10	83.33	0.043
5	Muscle integrity 0 - 3	2	S	5	41.67	
5	Muscle integrity 0 - 3	2.5	L	1	8.33	0.043
5	Muscle integrity 0 - 3	2.5	S	0	0.00	
5	Muscle integrity 0 - 3	3	L	1	8.33	0.043
5	Muscle integrity 0 - 3	3	S	6	50.00	
6	Fibroblasts 0 - 3 40x	0	L	1	8.33	0.453
6	Fibroblasts 0 - 3 40x	0	S	1	8.33	
6	Fibroblasts 0 - 3 40x	0.5	L	1	8.33	0.453
6	Fibroblasts 0 - 3 40x	0.5	S	0	0.00	

6	Fibroblasts 0 - 3 40x	1	L	8	66.67	0.453
6	Fibroblasts 0 - 3 40x	1	S	11	91.67	
6	Fibroblasts 0 - 3 40x	2	L	2	16.67	0.453
6	Fibroblasts 0 - 3 40x	2	S	0	0.00	
7	Leukocytes (lymphocytes & monocytes) 0 - 3	0	L	11	91.67	0.217
7	Leukocytes (lymphocytes & monocytes) 0 - 3	0	S	8	66.67	
7	Leukocytes (lymphocytes & monocytes) 0 - 3	0.5	L	1	8.33	0.217
7	Leukocytes (lymphocytes & monocytes) 0 - 3	0.5	S	1	8.33	
7	Leukocytes (lymphocytes & monocytes) 0 - 3	1	L	0	0.00	0.217
7	Leukocytes (lymphocytes & monocytes) 0 - 3	1	S	3	25.00	
8	Neutrophils (0 - 3)	0	L	12	100.00	
8	Neutrophils (0 - 3)	0	S	12	100.00	
9	Vasculature 20x (0-2)	0	L	5	41.67	0.126
9	Vasculature 20x (0-2)	0	S	7	58.33	
9	Vasculature 20x (0-2)	0.5	L	0	0.00	0.126
9	Vasculature 20x (0-2)	0.5	S	2	16.67	
9	Vasculature 20x (0-2)	1	L	7	58.33	0.126
9	Vasculature 20x (0-2)	1	S	3	25.00	
3.1	Macrophages near incision 0 - 4	1	L	6	50.00	0.680172
3.1	Macrophages near incision 0 - 4	1	S	8	66.67	
3.1	Macrophages near incision 0 - 4	2	L	6	50.00	
3.1	Macrophages near incision 0 - 4	2	S	4	33.33	

Table 1.0

Table 1.0 is a categorical classification of the multinomial variables by grade and method. This table contains the tabulation for the following parameters: macrophages near SIS suture, macrophages near scalpel incision, macrophages near commercial suture, muscle integrity, fibroblasts, leukocytes, neutrophils, and vasculature.

										95% CONFIDENCE INTERVALS	
SURGICAL PROCEDURE	GROWTH FACTOR	SEQ. NO.	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION	MEDIAN	MINIMUM VALUE	MAXIMUM VALUE	LOWER	UPPER
LASER	NO	10	Granulation Tissue	6	10.111	11.356	7.215	0.000	29.600	-1.806	22.028
LASER	YES	10	Granulation Tissue	6	24.133	16.539	22.004	5.165	53.792	6.776	41.489
SCALPEL	NO	10	Granulation Tissue	7	9.357	7.289	8.224	0.000	21.622	2.616	16.098
SCALPEL	YES	10	Granulation Tissue	5	15.647	6.459	17.258	5.455	22.506	7.627	23.667

Table 1.1

Table 1.1 is a univariate analysis that was performed for the following variables: surgical method, use of growth factors, and parameter based on the average intra-animal measurements of granulation tissue. This analysis showed that Growth factor (GF) has a positive influence on granulation tissue. The average granulation tissue approximately doubled in the groups that were under the influence of GF.

									95% CONFIDENCE INTERVALS	
SURGICAL PROCEDURE	SEQ. NO.	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION	MEDIAN	MINIMUM VALUE	MAXIMUM VALUE	LOWER	UPPER
LASER	10	Granulation Tissue	12	17.122	15.381	15.272	0.000	53.792	7.349	26.894
SCALPEL	10	Granulation Tissue	12	11.978	7.392	13.011	0.000	22.506	7.281	16.674

Table 1.2

Table 1.2 is a univariate analysis that was performed on the same variables as Table 1.1. The only difference is that Table 1.2 is independent of GF.

									95% CONFIDENCE INTERVALS	
GROWTH FACTOR	SEQ. NO.	PARAMETER	N	ARITHMETIC AVERAGE	STANDARD DEVIATION	MEDIAN	MINIMUM VALUE	MAXIMUM VALUE	LOWER	UPPER
NO	10	Granulation Tissue	13	9.705	8.969	7.959	0.000	29.600	4.285	15.125
YES	10	Granulation Tissue	11	20.275	13.156	18.944	5.165	53.792	11.437	29.114

Table 1.3

Table 1.3 is a univariate analysis that was performed regarding granulation tissue, growth factor was a parameter, but it was independent of the surgical method. The mean values were 9.705 and 20.275 in the no GF and GF groups, respectively.

SEQ. NO.	PARAMETER	SOURCE	DEGREES OF FREEDOM	TYPE III SUMS OF SQUARES	F-VALUE	PROBABILITY VALUE
10	Granulation Tissue	GROUP	3	863.98388686	2.3057478309	0.108

Table 2.1

Table 2.1 considered all 4 groups and analyzed them based on the intra-animal average granulation tissue. The p value was 0.108 and is highly suggestive of a significant difference among the 4 groups.

			GROUP			
SEQ. NO.	PARAMETER	SOURCE	A	B	C	D
10	Granulation Tissue	1	_	0.042	0.2243	0.028
10	Granulation Tissue	2	0.042	_	0.423	0.905
10	Granulation Tissue	3	0.2243	0.423	_	0.348
10	Granulation Tissue	4	0.0276	0.9046	0.3479	_

Table 2.2

Table 2.2 is a probability matrix that is comparing the arithmetic average among the groups for the production of granulation tissue.

SEQ. NO.	PARAMETER	RANK		MEAN	N	GROUP
10	Granulation Tissue		A	24.133	6.000	A
10	Granulation Tissue		A	15.647	5.000	C
10	Granulation Tissue		A	10.111	6.000	B
10	Granulation Tissue		A	9.357	7.000	D

Table 2.3

Table 2.3 is a Student-Newman-Keuls (SNK) test was preformed based on the one-factor analyses of variance for granulation tissue production. Based on the SNK test for granulation tissue it shows that there was really no difference amongst the 3 different groups based on the group mean square error term.

SEQ. NO.	PARAMETER	SOURCE	DEGREES OF FREEDOM	TYPE III SUMS OF SQUARES	F-VALUE	PROBABILITY VALUE
1	Macrophages near SIS	GROUP	3	0.000000		
2	Macrophages near commercial suture	GROUP	3	0.000000		
3	Macrophages near scalpel incision	GROUP	3	865.371429	60.9662	0.000
4	Macrophages near laser incision	GROUP	3	912.000000	101.3333	0.000
5	Muscle Integrity	GROUP	3	290.152381	3.5052	0.034
6	Fibroblasts	GROUP	3	55.101190	0.7012	0.562
7	Leukocytes	GROUP	3	158.967857	2.5321	0.086
8	Neutrophils	GROUP	3	0.000000		
9	Vasculature	GROUP	3	188.404762	1.7075	0.198
3.1	Macrophages near incision	GROUP	3	221.485714	2.3873	0.099

Table 3.1

Table 3.1 is an additional one-factor analyses of variance was preformed based on the ranked intra-animal median values to evaluate the effects of laser versus scalpel with and without the addition of GF on the presence of macrophages near SIS, near the commercial suture, near the laser incision, muscle integrity, number of fibroblasts, number of neutrophils, number of leukocytes, and number of blood vessels visible in each randomly surveyed 20X field.

SEQ. NO.	PARAMETER	SOURCE	A	B	C	D
1	Macrophages near SIS	1	_			
1	Macrophages near SIS	2		_		
1	Macrophages near SIS	3			_	
1	Macrophages near SIS	4				_
2	Macrophages near commercial suture	1	_			
2	Macrophages near commercial suture	2		_		
2	Macrophages near commercial suture	3			_	
2	Macrophages near commercial suture	4				_
3	Macrophages near scalpel incision	1	_	1	<.0001	<.0001
3	Macrophages near scalpel incision	2	1	_	<.0001	<.0001
3	Macrophages near scalpel incision	3	<.0001	<.0001	_	0.596
3	Macrophages near scalpel incision	4	<.0001	<.0001	0.5963	_
4	Macrophages near laser incision	1	_	0.0007	<.0001	<.0001
4	Macrophages near laser incision	2	0.0007	_	<.0001	<.0001
4	Macrophages near laser incision	3	<.0001	<.0001	_	1.000
4	Macrophages near laser incision	4	<.0001	<.0001	1	_
5	Muscle Integrity	1	_	0.2848	0.4351	0.088
5	Muscle Integrity	2	0.2848	_	0.804	0.008
5	Muscle Integrity	3	0.4351	0.804	_	0.020
5	Muscle Integrity	4	0.0882	0.0082	0.0201	_
6	Fibroblasts	1	_	0.219	0.5608	0.236
6	Fibroblasts	2	0.219	_	0.5433	0.924
6	Fibroblasts	3	0.5608	0.5433	_	0.590
6	Fibroblasts	4	0.2364	0.9244	0.5896	_
7	Leukocytes	1	_	0.5152	0.0159	0.474
7	Leukocytes	2	0.5152	_	0.0589	0.967
7	Leukocytes	3	0.0159	0.0589	_	0.056
7	Leukocytes	4	0.474	0.9668	0.0557	_
8	Neutrophils	1	_			
8	Neutrophils	2		_		
8	Neutrophils	3			_	
8	Neutrophils	4				_
9	Vasculature	1	_	0.0782	0.9286	0.911
9	Vasculature	2	0.0782	_	0.0775	0.085
9	Vasculature	3	0.9286	0.0775	_	0.843
9	Vasculature	4	0.9112	0.0848	0.8426	_

3.1	Macrophages near incision	1	_	0.0216	0.4155	0.649
3.1	Macrophages near incision	2	0.0216	_	0.1382	0.046
3.1	Macrophages near incision	3	0.4155	0.1382	_	0.678
3.1	Macrophages near incision	4	0.6493	0.0463	0.6781	_

Table 3.2

A probability matrix comparing the intra-animal arithmetic averages among the groups from the one-factor analyses of variance tests based on the ranked intra-animal arithmetic average values by parameter (macrophages near SIS, near commercial suture, near scalpel incision, muscle integrity, number of fibroblasts, number of neutrophils, number of leukocytes, and number of blood vessels visible in each randomly surveyed 20X image).

SEQ. NO.	PARAMETER	RANK		MEAN	N	GROUP
1	Macrophages near SIS		A	12.5	6.000	A
1	Macrophages near SIS		A	12.5	6.000	B
1	Macrophages near SIS		A	12.5	5.000	C
1	Macrophages near SIS		A	12.5	7.000	D
2	Macrophages near commercial suture		A	12.5	6.000	A
2	Macrophages near commercial suture		A	12.5	6.000	B
2	Macrophages near commercial suture		A	12.5	5.000	C
2	Macrophages near commercial suture		A	12.5	7.000	D
3	Macrophages near scalpel incision		A	18.9	5.000	C
3	Macrophages near scalpel incision		A	18.21	7.000	D
3	Macrophages near scalpel incision		B	6.5	6.000	A
3	Macrophages near scalpel incision		B	6.5	6.000	B
4	Macrophages near laser incision		A	20.5	6.000	B
4	Macrophages near laser incision		B	16.5	6.000	A
4	Macrophages near laser incision		C	6.5	5.000	C
4	Macrophages near laser incision		C	6.5	7.000	D
5	Muscle Integrity	A		17.57	7.000	D
5	Muscle Integrity	A	B	12.33	6.000	A
5	Muscle Integrity		B	9.8	5.000	C
5	Muscle Integrity		B	9	6.000	B
6	Fibroblasts		A	14.83	6.000	A
6	Fibroblasts		A	13	5.000	C
6	Fibroblasts		A	11.36	7.000	D
6	Fibroblasts		A	11.08	6.000	B
7	Leukocytes		A	17.3	5.000	C
7	Leukocytes		A	11.86	7.000	D

7	Leukocytes		A	11.75	6.000	B
7	Leukocytes		A	10	6.000	A
8	Neutrophils		A	12.5	6.000	A
8	Neutrophils		A	12.5	6.000	B
8	Neutrophils		A	12.5	5.000	C
8	Neutrophils		A	12.5	7.000	D
9	Vasculature		A	17.33	6.000	B
9	Vasculature		A	11.21	7.000	D
9	Vasculature		A	10.83	6.000	A
9	Vasculature		A	10.5	5.000	C
3.1	Macrophages near incision		A	17.5	6.000	B
3.1	Macrophages near incision		A	12.3	5.000	C
3.1	Macrophages near incision		A	10.93	7.000	D
3.1	Macrophages near incision		A	9.5	6.000	A

Table 3.3

A Student-Newman-Keuls test from the one-factor (group) analysis of variance tests based on the ranked intra-animal median average values by parameter (macrophages near SIS, near commercial suture, near scalpel incision, muscle integrity, number of fibroblasts, number of neutrophils, number of leukocytes, and number of blood vessels visible in each randomly surveyed 20X image) was also completed.

SEQ. NO.	PARAMETER	SOURCE	DEGREES OF FREEDOM	TYPE III SUMS OF SQUARES	F-VALUE	PROBABILITY VALUE
10	Granulation Tissue	INTERVENTION	1	158.78204432	1.0905155328	0.308

Table 4.1

A one-factor analysis of variance tests based on the intra-animal arithmetic average values by parameter was done on all the groups using the parameter of laser vs scalpel when looking at granulation tissue.

Chapter 4. Discussion

The goals of this light microscopy study were to histologically compare carbon dioxide laser versus scalpel incisions. SIS sutures versus commercial sutures, and the effect of NGF or no added NGF. This study was performed in conjunction with two other studies that utilized the same group of experimental animals. One of the studies compared healing of the epidermis following scalpel or CO2 laser incision alone or with added growth factor. The other study compared kinesthetic changes post-operatively in these four experimental groups. Where appropriate the results of the present study will be discussed in conjunction with the results of the other two studies.

The analysis of gastrocnemius muscle parameters that were found to be statistically significant included muscle integrity, quantity of vasculature, width of granulation tissue, number of macrophages in proximity to the incision, and prevalence of leukocytes.

Muscle Healing

Muscle integrity as well as the orientation of contractile components is an important factor in normal muscle function. The experimental groups with added growth factor resulted in a higher degree of muscle integrity. Muscle integrity was measured by visualizing the fibers/nuclie/myofilaments at 20X in randomly selected fields and rating the level of organization on a scale of 0-3, ‘0’ for “disorganized,” through ‘3’ for “highly organized.”

Previous studies have shown that muscle regeneration has increased significantly with the addition of growth factor (7). Growth factors have been shown to

accelerate the normal healing process, but also to aid in healing of the previously incurable wounds (23). Other studies have shown that neutralizing inhibitory growth factors rather than adding additional growth factors may be beneficial (2). There is some disagreement whether neutralizing inhibitory growth factors instead of adding growth factors may be beneficial since some results have shown that factors that inhibit proliferation and differentiation of muscle prevent healing and regeneration (1,2). However, the majority theory regarding the contribution of growth factors to muscle regeneration is that they each have a specific role in recovery and healing of muscle (1,2,3,7,23).

Vasculature

Angiogenesis is critical for proper wound healing, especially in the muscle which has a high demand for oxygen and produces a significant amount of metabolic end products that musce be removed in order that the muscle heal effectively. Without adequate delivery of nutrients, (including oxygen) to the tissues and removal of metabolic products the muscle would not be able to heal normally. This was one of the focuses of this study and angiogenesis was indirectly assessed by examining the presence or absence of vasculature in randomly chosen fields. The resulting analyses showed that the scalpel group with added growth factor produced the highest density of blood vessels followed by the scalpel group without added growth factor, then laser group with added growth factor, and lastly the laser group without growth factor.

A study by Skully and Hughes has shown that the trauma caused by the use of a scalpel stimulates cytokines, growth factors and other properties that aid in promoting

angiogenesis to a greater extent than the use of the laser (31). Another study suggested that trauma to a muscle or organ stimulates bone-derived endothelial progenitor cell release, which significantly increases neovascularization in the injured region (32). There also have been several studies that demonstrated basement membrane proteins contribute to angiogenesis; this supports the results of the study reported here since the growth factor that was used is also found in the extracellular matrix of the muscle (33).

The greatest increase in skin vascularity observed in the companion study occurred in the laser with GF group whereas the greatest increase in muscle vascularity was found in the scalpel group with added GF (45). This discrepancy in the density of blood vessels may be due ot the different distribution of these structures in skin in and muscle, or it may be due to the fact that muscle is a longitudinally oriented tissue with blood vessels entwined around the muscle fibers, whereas skin is not similarly orients and the distribution of blood vessels is not uniform in the different epidermal layers. Alternatively, the inherent properties of these two tissues may have resulted in different responses to the two surgical modalities as well as to added growth factor. Unfortunately, there are a limited number of reports in the literature that compare the degree of vascularity associated with scalpel versus laser incisions, and most of these studies are focused on epidermal responses rather than those of muscle so it is difficult to compare them to the present study.

Granulation Tissue

Although the production of granulation tissue is part of the normal healing process, a prolonged inflammatory response with a proliferation of granulation tissue can interfere with the normal wound healing process and allow for an increase susceptibility to infection (37,38). The width of the granulation layer was measured as an indicator of the extent of the foreign body reaction among the different treatment groups.

The measurements were performed on histological images obtained from random fields surrounding the suture holes in each of the experimental groups. The resulting analyses demonstrated that the laser group with added growth factor produced the greatest width of granulation tissue followed by the scalpel group with added growth factor, then the laser group without added growth factor, and finally the scalpel group without growth factor.

The results indicate that both the use of the laser and the addition of growth factor contributed significantly to an increase in granulation tissue, and may be related to laser dose. The selection of an appropriate laser dose was based upon preliminary examination of responses obtained using chicken cadavers. Although the tissues of this species are similar, they do not identically replicate the muscle properties of the rat or its water content and it is likely that the laser dose selected could have been better optimized by laser dose titration studies that utilized rat tissues. However, this was a proof of concept study which limited the number of animals that could be included and thus precluded using this approach. The laser dose selected was based upon

preliminary chicken tissue studies as well as calculations based upon other studies reported in the literature that utilized the CO_2 laser (40). Another related factor that may be bearing upon these results was reported in a study by Roeder et al who found that "by delivering rapidly overlapping pulses and scans, residual thermal damage and cell death depth were increased as much as 100% over areas without immediate overlap of laser impacts" (38). Significantly the study here employed a "four-pass" technique to produce the full thickness muscle incisions. Another related study reported an increase in granulation tissue resulted from the failure to allow adequate cooling of the tissue prior to sequential passes thus producing an additive thermal effect (39).

The results for width of epidermal granulation tissue in the companion study to this study found similar results, i.e. the greatest increase in width of granulation tissue occurred in the laser with GF group and the narrowest of granulation tissue occurred in the scalpel group without added GF (46).

Macrophages

In response to tissue injury, mononuclear phagocytes (macrophage progenitors) migrate from the venous system to the site of tissue injury and initiate the inflammatory response. Deployment of monocytes/macrophages to the site of injury peaks as the number of neutrophils decline. Once present, they differentiate into mature macrophages. After activation, macrophages are the main source of growth factors and cytokines that modulate tissue repair. Wound healing following a surgical incision similarly to other types of tissue injury results in an inflammatory response.

Macrophages in proximity to the laser and scalpel incisions were visualized and recorded by counting the number of cells in randomly selected fields at 40x magnification and ranking the images on a scale of 0 to 3 in which “1” was equal to a count of 1 to 4, “2” was equal to a count of 5 to 10, “3” was equal to a count of more than 10 cells.

The prevalence of macrophages as well as the number of leukocytes were used to determine the degree of the inflammatory response for each experimental group. The resulting analyses showed that the laser group with added growth factor produced the greatest number of macrophages followed by the laser group without added growth, then the scalpel group with added growth factor, and finally the scalpel group without added growth factor. These results as well as those obtained by measuring the width of granulation tissue support the Manolis et al, postulation that laser stimulates production of endogenous growth factors, and granulation tissue as a reflection of the extent of the inflammatory response (32).

When tissues are inured and vascularity is interrupted it leads to a hypoxic state with the release of increasing numbers of macrophages, which in turn release and active angiogenesis factor (43). This also supports the results found here of an increased vascular density in the laser with growth factor group and the scalpel with added growth factor group both of which had a greater vascular density in the muscle in comparison to the experimental groups.

Since the inflammatory phase of wound repair is characterized by a migration of macrophages, future studies in which this influx is studied at sequential times may more

closely characterize the wound healing process in skeletal muscle. This could be accomplished through the use of the nitroblue tetrazolium test, which is sensitive to the presence of alkaline phosphatase and therefore can determine the amount of phagocytosis taking place by the macrophages (44).

Leukocytes

Leukocyte infiltration along with monocytes/macrophages is hallmark of the inflammatory phase. Leukocytes were analyzed by visualizing and recording the number of cells in randomly selected fields at a magnification of 40X and ranking the results on a scale of 0 to 3 in which “1” was equal to a count of 1 to 4, “2” of 5 to 10, “3”, a count of more than 10 cells.

The results suggested that leukocytes were to be increased in all experimental groups. These analyses were highly suggestive of statistical significance (p value of 0.086), but more studies need to be done to confirm or refute this trend in the data. The analysis is confounded by the fact that there are7 different types of leukocytes (neutrophils, eosinophils, basophils, monocytes, macrophages, lymphocytes, and dendritic cells) and this study did not selectively analyze each population. Future studies that discriminate between neutrophils and monocytes would be insightful since these white cells are most active in wound healing. Neutrophils especially, are a highly abundant blood-cell population in the circulation, and quite a significant number of neutrophils collect passively at wound sites in the blood clot as a result of blood-vessel disruption and the concomitant extravasation of blood constituents; after the “passive” extravasation they migrate immediately to the wound surface to aid in healing (45).

Confounding Variables

Operator Error: none of the investigators were blinded to the treatment of the animals as each investigator was assigned their own responsibilities: supplement injections of GF; gait analysis; application of antibacterial ointment; twice-a-day animal monitoring for the first 48 hrs post-operatively and once a day for the next 5 days; euthanization.

Growth factor distribution: tagging the GF with a marker would have identified the site of integration in the muscle. Since the incisions healed well, it was a determination of whether or not the GF was actually injected into the incision or just near the incision was not possible.

Additional analyses: Post surgical mediators of stress such as cytokines and chemokines could have been evaluated to further correlate their release with the inflammatory response.

Suggested Future Studies

Study Design: Varying the endpoints for euthanasia within the different treatment groups would allow for the observation of wound healing at different stages throughout the healing process in order to better compare experimental variables at different time points.

The choice of the rats as an experimental animal is supported by a large body of literature based upon the examination of various aspects of rat skeletal muscle. However, there still remains the assumption that factors such as weight-bearing, post transcriptional transformation in fiber isoforms, and other differences in responsiveness

to experimental variables are similar. It remains to be resolved to what extent the results obtained using the animal model are transferrable to human skeletal muscle healing.

Additional Analyses: Post surgical mediators of stress such as cytokines and chemokines could be evaluated to further correlate their release with the inflammatory response.

Different types of GF, such as vascular endothelial growth factor (VEGF) or fibroblastic growth factor (FGF), could be compared to determine if results could be obtained that are similar to what was found in this study, or to differentiate between their effects, since each has a unique primary function. The possibility also may exist that they produce convergent response in the muscle tissue.

Alternate Methodologies: Impregnating GF into the SIS suture material prior to surgery may help to further localize the distribution of GF in the muscle and influence both the extent of granulation tissue and vascularity.

Other Considerations: Cutaneous wound healing following the use of laser or scalpel incision modalities is better described especially as affected by GF, than is the response of skeletal muscle. This line of research has been driven in part by the quest for more aesthetic outcomes. However, skeletal muscle function, especially where it affects limb function has a large impact on the quality of life at all stages of a patients life. Focused research that targets post-operative healing is needed in order to develop new strategies to reduce levels of disability following surgical interventions. One example of such a strategy that might be applied to post-surgical muscle healing is

currently being utilitzed in the treatment of stage 4 diabetic ulcers that have a skeletal muscle component (Becaplermin, REGRANEX® 0.01% gel) (34,35,36).

This research will lead to improvements in skeletal muscle wound healing that can be adopted by emergency medicine physicians and surgeons, and every other physician who has to plays a role in wound healing and management of their patients.

Appendix

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